THE OVUM OF THE NINE-BANDED ARMADILLO. GROWTH OF THE OVOCYTES, MATURATION AND FERTILIZATION.

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I. INTRODUCTION.

Interest in the armadillo ovum is due chiefly to its unique capacity for polyembryonic development. As has been shown (Newman and Patterson, '10) the ovum develops throughout the early stages of the embryonic period as a single blastodermic vesicle and, only after the differentiation of the primary germ layers, divides visibly into four embryonic primordia. That the four embryos of a litter are always of the same sex has also aroused the interest of biologists, especially those engaged in researches on the problem of sex determination. Since in the armadillo sex would seem to be predetermined in the undivided oosperm an examination of the cytology of both male and female sex cells should be made.

Many conjectures as to the underlying cause of polyembryony in the armadillo have no doubt been made by all who have taken an interest in the phenomenon. Among those that have been most commonly suggested are the following:

1. On the basis of a cytological examination of one pair of ovaries Rosner ('01) concluded that four adjacent follicles fuse in such a way that four eggs are thrown into a single follicular cavity; on the rupture of this compound follicle four eggs are discharged simultaneously, descend the fallopian tube held together in a mass by means of their discus proligerus cells, become fertilized, undergo cleavage and come to a common point of attachment in the uterus; subsequently the contiguous walls of the four blastocysts atrophy and a single vesicular chorion is produced. According to Rosner then polyembryony does not exist, but merely the appearance of polyembryony, due to an early fusion of four blastodermic vesicles.

2. It has been suggested that the ovum might give off two large polar bodies and that the first of these might divide, thus producing four potential ova within one zona. These would be separately fertilized and would produce a morula apparently simple but actually quadruple. Subsequently the four embryonic components would segregate themselves and produce the quadruplets. This view also denies the reality of polyembryony.

3. There might occur an early fusion of four ovogonia or ovocytes to form a tetra-nuclear germ cell, which would be fertilized by as many spermatozoa as there were female pronuclei and thus give rise to a quadruple embryonic vesicle.

4. The two maturations might occur within the cytoplasm of the egg, without the extrusion of polar bodies. We would have in this case an egg with four pronuclei which, when fertilized with four spermatozoa, would be able, conceivably, to produce four embryos.

5. There might occur two successive parthenogenetic divisions of the female pronucleus, prior to fertilization, which would require four spermatozoa for their fertilization and would thus account for the observed conditions.

6. The cytoplasmic materials of the ovum might be physiologically isolated into quarters during some period of the ovarian history. Such a condition might conceivably foreshadow the actual isolation of embryonic primordia as it occurs at a fairly early period of embryonic development.

7. The cause of specific polyembryony may lie in factors

strictly external to ovum, among which one of the most probable is in some way associated with the bilaterality of the uterus. A discussion of this possibility would be foreign to the topic in hand and must be postponed for subsequent treatment.

The first five possible explanations will probably appear to the reader to be without foundation and far-fetched. Every one, however, has been offered by serious-minded biologists. The majority of these explanations have already been shown to be untenable, some require further refutation. It is one of the purposes of this paper to remove the latter from further consideration. There is a small amount of evidence in favor of the sixth suggestion, but it is far from convincing. So it would appear that the stimulus to specific polyembryony must be looked for in some external factors, the character of which we are not prepared to discuss at present. This being the case a study of the history of the female germ cells can furnish only negative evidence on the main question at issue and would therefore lack the inherent interest that usually attaches to positive results, were it not that a considerable number of interesting, and, I believe, important facts, apparently quite unrelated to the phenomenon of polyembryony, have come to light. These facts are therefore presented partly to pave the way for further cytological studies but principally because they appear to possess a value quite independent of any of the general problems so far suggested by studies of the armadillo.

It is shown in this paper that the armadillo ovum bears a remarkably close resemblance to that of *Dasyurus*, the native marsupial cat of Tasmania, described by Hill ('10). In both *Dasyurus* and *Tatu* the ovum, at maturity, exhibits an inverted "telolecithal" condition. The genesis of this peculiar state of affairs is traced through the growth period of the ovocyte and incidentally a description of the parallel development of follicle, ovocyte and germinal vesicle during this extensive period is presented in the belief that this phase of ovogenesis has been too largely neglected by students of the maturation and fertilization of the mammalian ovum. It will be noted also that the armadillo ovocyte is especially favorable for the study of chromosomal behavior during the maturation processes and that it is possible

with some assurance to enumerate the elements of the chromosome complex. This should furnish a useful companion study to that of the spermatogenesis which is being worked out by Dr. J. T. Patterson. Finally, the facts here presented serve to banish any hesitation that may at any time have been entertained as to the validity of the assumptions upon which are based the studies of the predeterminative and epigenetic factors concerned in the development of the definitive characters of the armadillo quadruplets, studies which were outlined in a former publication (Newman and Patterson, '11) and which are at present being carried on with a much more adequate collection of material.

II. Literature on Mammalian Ovogenesis.

Considered solely as a contribution to our knowledge of the maturation and fertilization processes of mammalian ova the present study would be well worth presentation owing to the fact that the work in this field has been confined to three orders of mammals, Rodentia, Cheiroptera and Carnivora. Nothing is known of the conditions in any Edentate. It is a pleasure then to add to this brief list not only an additional order but one in which the ovum is of a type more primitive than any previously noted for *Eutheria*.

Our knowledge of ovogenesis in the Cheiroptera is limited to one species, *Vesperugo noctula*, described by O. Van der Stricht in 1909. The only representative of the Carnivora which has received adequate attention is the domestic cat, the maturation and fertilization of which have been recently worked out in detail by Longley ('11). The rodents however, have furnished the basis for numerous elaborate studies. Conditions in the guinea-pig, the rat and the mouse are known in detail and especially is this the case with the mouse, upon which no less than eight investigations have been published. All of this rather voluminous literature has been recently reviewed by several authors and for details the reader is referred to the papers of Kirkham ('07), Sobotta and Burckard ('10), Long and Mark ('11) and Longley ('11).

We have then adequate accounts of the ovogenesis of only five species of mammals: the bat, the cat, the guinea-pig, the rat

and the mouse. Of these all but the bat are domesticated forms; so the armadillo is the second species of wild mammal whose ovogenesis has been investigated.

Longley ('11) points out very pertinently that the failure on the part of investigators to secure material for the study of ovogenesis in the higher mammals is due partly to the difficulty of procuring the eggs of these forms in the conditions needed and partly to the fact that the ovaries of large animals are too bulky for convenient investigation, involving as they do a study of serial sections of a comparatively enormous mass of tissue.

III. Material and Method.

In the pursuit of the study of the maturation and fertilization processes of wild mammals two courses are open to the investigator. He may breed them in captivity, a precarious and not often successful undertaking involving the killing of many animals tamed at great pains. The only remaining course of action is that which has been resorted to in the present investigation, namely, to rely upon the chance collection of favorable stages in the ovaries or fallopian tubes of freshly captured females during the period of œstus.

In the case of the armadillo of Texas very serious difficulties are encountered in keeping the animals and breeding them in captivity. In the first place they appear to breed but once a year and would therefore have to be kept in considerable numbers for a long time in order that an adequate collection of stages could be made. Experience has shown that the animals are extremely difficult to domesticate. They need much territory for the exercise of their normal functions and apparently would breed only if allowed to burrow in the ground as is their custom. In addition to these obstacles to rearing, the animals, as they have come under my observation, are almost invariably badly infested with flesh parasites that rapidly gain the ascendency if the animals are subjected to conditions somewhat less favorable than the normal.

In view of these conditions I have been forced to rely on serial sections of ovaries and the attached fallopian tubes for my studies of maturation and fertilization. The former process is,

I am convinced, well illustrated in the material at hand; the latter has been found only in one case, but this has all the earmarks of a normal fertilization stage and is therefore accepted as typical for the species, pending further evidence which may or may not be forthcoming.

During three years material for this paper has been collected and studied. Ovaries of adult and young females have been fixed in various fluids and studied at all stages of the sexual cycle. As a rule the best stages have been obtained from the ovaries of large females taken at the height of the mating season. Ovaries of pregnant females show little of interest in this connection.

Out of a considerable variety of fixing agents used it soon became apparent that by far the most efficient for nearly every purpose was Zenker's fluid. Fleming's, Gilson's, Bouin's, Petrunkewitsch's and formalin Zenker gave uniformly less satisfactory results and were not used after the first few trials. For the study of vitellogenesis the ovaries were fixed in 10 per cent. neutral formalin and favorable free-hand sections were stained in Sudan III. This material, when counterstained with a weak solution of methyl green was also best for measurements of ovocytes, as there was practically no shrinkage.

A variety of staining processes gave satisfaction, but the best for both nuclear and cytoplasmic details proved to be Bensley's copper chrome hæmatoxylin process. This stain gives as sharp definition of chromosomes as does Haidenhain's iron hæmatoxylin method and in addition stains acromatic nuclear materials and cytoplasmic structures admirably. On account of the standard character of the iron hæmatoxylin technique, however, this stain was used throughout as a control. For certain special points several other staining combinations were employed, notably toluidin blue and acid fuchsin, neutral safranin and acid violet, and thyonin and erythrosin. These served to bring out certain differentiations that could be discovered only by their aid.

IV. Scope and Outline of Observations.

The present study begins with a consideration of conditions found in ovocytes at the beginning of the period of growth, just before they have acquired primordial follicles. It appears necessary to begin the study of maturation thus early partly because the prophases of maturation appear to be well under way at this period and partly because the development of the follicle and the relations of the ovocytes to the follicle are of fundamental interest in connection with the investigation into the causes of polyembryony. The growth of the ovocyte is accompanied by growth and modification of the follicle and the development of both culminates in a condition which would normally be followed by ovulation. Failure of ovulation, however, is the fate of the vast majority of developing ova, owing partly to their position in the ovary but chiefly to the influence of pregnancy, the occurrence of which inhibits further ovulation. These ova which have reached full size and are in every way mature and ready for ovulation, occasionally complete the process of maturation in a manner identical with that which normally occurs only in ova which have been fertilized, and under some conditions develop parthenogenetically through the cleavage period, as I have determined quite conclusively. In lieu of any data concerning the completion of the second maturation division in tube eggs, it is considered a legitimate procedure to substitute an account of the condition seen in these ovarian ova in which the maturation process has proceeded beyond the stage ordinarily seen in such ova. That this is a justifiable use of material is shown by the fact that in all species where both the normal process of maturation, and that seen under conditions identical with those just indicated, have been studied, there has been found no essential difference between them.

The study then may be conveniently divided into two parts, the first dealing with processes taking place in normal follicles up to a period when ovulation would normally take place, and the second with the completion of the maturation in follicles undergoing the early stages of follicular atresia. In all other species where the facts are known ovulation occurs during the

second maturation division, after the first polar body has been extruded. Stages up to this period are fairly numerous in the present material, but there are only two examples of ovarian ova completing the second maturation. Without further preliminaries then it will be understood that all stages of the process as here described are normal with the exception of those described and shown in Figs. 42 and 43.

It seems best to describe first the stages of follicular growth, for it is very convenient to refer various stages of the developing ovocyte to certain figured stages of follicular differentiation. The growth period of the ovocyte, from the condition when it is without a follicle to the period of maturity, is next taken up, and incidentally the process of vitellogenesis and its consequences receives attention. For the sake of completeness and as a transition to the next study the matter of nuclear growth as compared with cell growth is considered. Finally the nuclear changes, principally those concerned with the formation of the chromosomes and the acromatic structures of the maturation spindles, are described in detail since chief interest appears to center upon these changes rather than upon any transformations or reorganization of the cytoplasm.

V. Development of the Graafian Follicle.

There appears to be nothing especially specific in the process of folliculogenesis as it occurs in the armadillo. Comparisons have been made stage for stage with that of the cat, the ovaries of which are of about the same size as those of the armadillo, and only very minor differences have been noted. This fact, in itself a matter of no particular moment, gains importance when it is remembered that Rosner ('01) attempted to explain away polyembryony on the basis of a very peculiar sort of fusion of adjacent follicles. The following account will serve finally to set at rest any misconceptions that may have been engendered by Rosner's unfortunate account. In addition we shall be afforded a sort of convenient time schedule upon which to hang the descriptions of the other processes with which the present study is concerned; for we shall be able to refer any particular phase of ovocytic or nuclear development to some definite stage

of follicular growth. Full grown ovocytes, for example, are invariably to be found in follicles of stage 10 (Fig. 10), while mature or maturing ovocytes are found only in follicles of type 11 (Fig. 11). It is fortunate for the student of ovogenesis that the condition of the follicle furnishes such an accurate index of the more important ovocytic changes, since it enables him to search through a large amount of material with low powers of the microscope and to detect readily certain follicles which he may examine with the assurance that he will find the desired stages of ovocytic or nuclear development. Hence the following very brief account of folliculogenesis is offered largely for the purpose of rendering the subsequent ovocytic and nuclear history more easily followed by the reader.

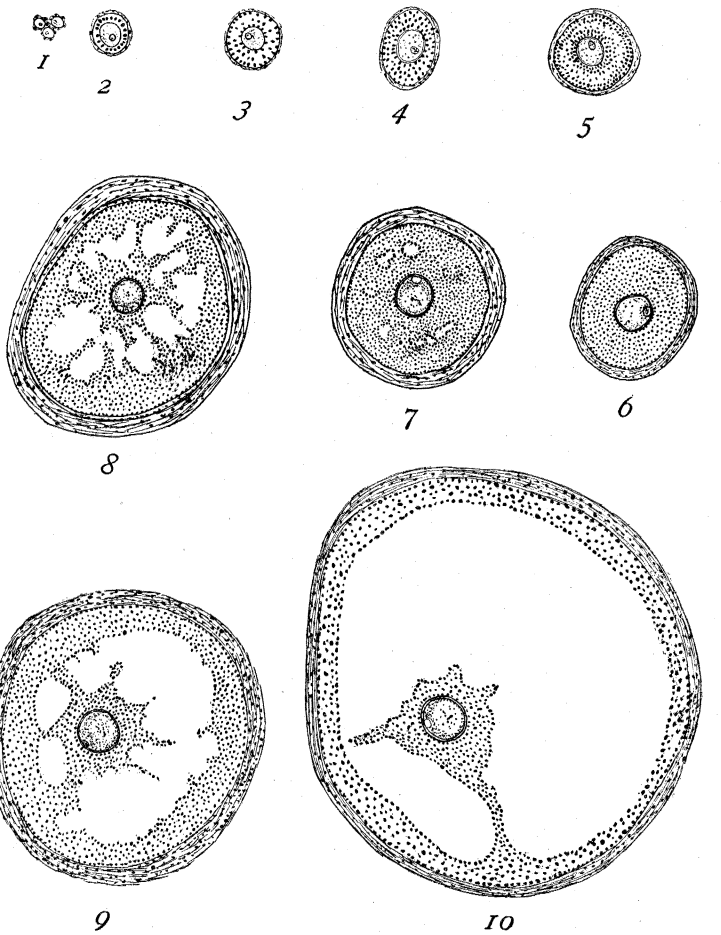
Stage 1 (*Fig. 1*).—The primordial follicle is just in process of establishment. Frequently nests of young ovocytes are found to be surrounded by primary follicular cells, giving the impression of several ovocytes in a single follicle. This is not really a pluriovular follicle but simply a stage prior to the completion of the establishment of the true primordial follicle. It is, however, positively the only condition in the entire process of ovogenesis in which I have found anything that might be looked upon as a fusion of follicles. The diameter of the follicle at the time when the ovocyte is surrounded with follicle cells averages about .03 mm.

Stage 2 (*Fig. 2*).—The single layer of primary follicle cells takes the form of a simple cubical epithelium, sharply cut off from the capsule of stroma cells. Both ovocyte and follicle cells have grown considerably, for the average diameter of follicles of this stage is about .1 mm.

Stage 3 (*Fig. 3*).—The simple epithelium of the follicle becomes compound. The figure shows a three-layered condition. Follicles at this stage have an average diameter of about .15 mm.

Stage 4 (*Fig. 4*).—There is a strong tendency at this stage for the follicle to become elongated on account of the more rapid proliferation of the cells at the two ends. This condition is usually accompanied by an elongation of the ovocyte as shown in Fig. 15. This phenomenon is of such frequent occurrence

that I am inclined to offer the tentative suggestion that a bilaterality of the ovocyte might be initiated here, which under certain conditions might produce a physiological isolation of the two halves of the germ cell and might account for the production



FIGS. 1 to 10 (inclusive) show ten stages in the development of the definitive follicle (×50).

of the paired embryonic primordia that are such noteworthy features of the early development as shown recently in Patterson's photographs.[1] Follicles at this stage measure on the average about .15 × .2 mm.

[1] These photographs were exhibited at the Urbana meeting of the Central Branch of the American Society of Zoölogists, held in April 1912.

Stage 5 (*Fig. 5*).—Later stages show as a rule a more or less complete loss of the elongated condition. The figure is a good example of a somewhat more advanced condition, the diameter of such follicles averaging about .22 mm.

Stage 6 (*Fig. 6*).—Here we have about the maximum development of the solid follicle, before a disintegration of follicular cells begins to give rise to a lumen. The compound epithelium is from five to seven layers thick and the capsule of stroma cells is more sharply defined than ever. Such follicles have an average diameter of about .3 mm.

Stage 7 (*Fig. 7*).—At this time through the cytolysis of some of the components of the epithelium various fluid-filled cavities appear midway between the ovocyte and the periphery of the follicle. Average diameter, about .35 mm.

Stage 8 (*Fig. 8*).—At this stage lumen formation has made considerable progress and the follicle cells may be considered as forming two zones: a zone around the ovocyte, which is destined to form the discus proligerus and a zone occupying a peripheral position. The intervening cavity is filled with follicular fluid and cell fragments. Average diameter of such follicles, about .5 mm.

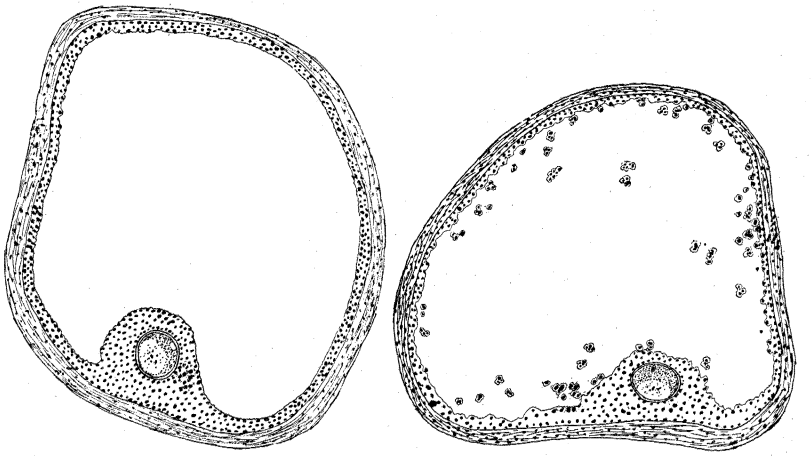
Stage 9 (*Fig. 9*).—At this time there is evinced a strong tendency for the ovocyte, with its zone of follicular cells, to occupy an excentric position due to the breaking away of the connecting strands of follicle cells on one side and the thickening by contraction of the others. This is evidently a step in the establishment of the definitive discus proligerus. Average diameter, .6 mm.

Stage 10 (*Fig. 10*).—Here we have another step in the development of the discus proligerus. Many of the largest follicles found have been in this stage of development. Average diameter, about 1 mm.

Stage 11 (*Fig. 11*).—This is what might be termed the definitive follicle. The discus proligerus is in the form of a smooth mound of follicle cells projecting into the lumen from one side of the follicular wall. The remaining part of the follicle is lined with a thin smooth sheath of follicle cells. There is a great deal of variation in the size and shape of the definitive follicle, many being flattened or otherwise distorted by the presence of various

obstructions such as older follicles or dense masses of stroma cells. The average diameter of the less distorted definitive follicles is about 1 mm.

Stage 12 (*Fig. 12*).—The conditions shown in this figure are readily recognized as those typical of follicles shortly after the onset of follicular atresia. The characteristic symptoms of this



11 *12*

FIG. 11. A definitive follicle with maturing ovocyte located in the mound-like discus proligerus. The lumen is filled with a clear fluid. A membranous capsule composed of stroma cells surrounds the follicular wall (×50).

FIG. 12. A follicle fully mature and entering upon process of follicular atresia. The cells of the discus proligerus and of the wall surrounding the lumen are beginning to disintegrate and to wander into the lumen (×50).

process are seen in the less deeply stained follicle cells and in the tendency of the latter to wander into the lumen. Such follicles frequently show a slight or marked diminution in size, due probably to a resorption of the follicular fluid. It is in such follicles that one finds the completion of the second maturation division, a process which does not normally take place until after ovulation.

In referring stages in the development of the ovocyte or nucleus to their appropriate follicular stages it will be convenient to remember that the first twelve figures have numbers corresponding to the twelve stages of follicular development; hence it will be necessary to refer only to stage 4, 7 or 10, with the understanding that these stages are illustrated in Figs. 4, 7 and 10.

VI. Development of the Ovocyte with Especial Reference to Vitellogenesis and the Comparative Rate of Growth of Nucleus and Cytoplasm.

The ovocytes in primordial follicles (stage 1) are comparatively small cells with homogeneous cytoplasm and large nuclei, the average diameter of ten typical cells being .033 mm. and that of their nuclei .015 mm. At the beginning of the growth period then the cell diameter is only about twice that of the nucleus.

Soon after the formation of the primordial follicle, before any marked growth of the cell has occurred, distinct spherules of fatty material are distinctly visible in preparations fixed in 10 per cent. formalin and stained in Sudan III. Evidently yolk metabolism has begun at this stage. These spherules are so brightly stained with the Sudan that no other interpretation of their character is admissible. They appear excentrically, being confined to one side of the nucleus, thus indicating an early cell polarity. The average diameter of such ovocytes is about .35 mm. and that of their nuclei about .17 mm. The proportionate size of cell and nucleus, therefore, has not been materially altered. Such a cell is shown in Fig. 14, which was drawn from an ovocyte somewhat below the average in size, occupying a follicle in a condition between stages 1 and 2.

The changes in the ovocyte as found during follicular stages 2, 3 and 4 culminate in a condition shown in Fig. 15, where the cell is frequently elongated, showing polarity and bilaterality. The yolk spherules are very distinct and abundant and are confined to the pole opposite to that occupied by the nucleus. The average largest diameter of such cells is about .08 mm. and that of their nuclei about .025 mm. It will be noted that the cytoplasmic mass has increased relatively much more rapidly than has that of the nucleus, although the latter has doubled its diameter and increased its mass several times. The zona pelucida is present as a comparatively thin but dense layer, which shows evidences of having been laid down as a mesh-work of fibrous material secreted by the basal portions of the follicle cells.

During follicular stages 5, 6 and 7 a curious change occurs in connection with the process of vitellogenesis. There is a gradual

disappearance of the yolk spherules (which earlier constituted such a marked feature of the cytoplasm) culminating in the condition shown in Fig. 16, in which the protoplasm of the ovocyte has acquired a secondary homogeneous structure, with a coarsely alveolar appearance. There are present scarcely any discrete fatty particles, but the whole cytoplasmic mass assumes a

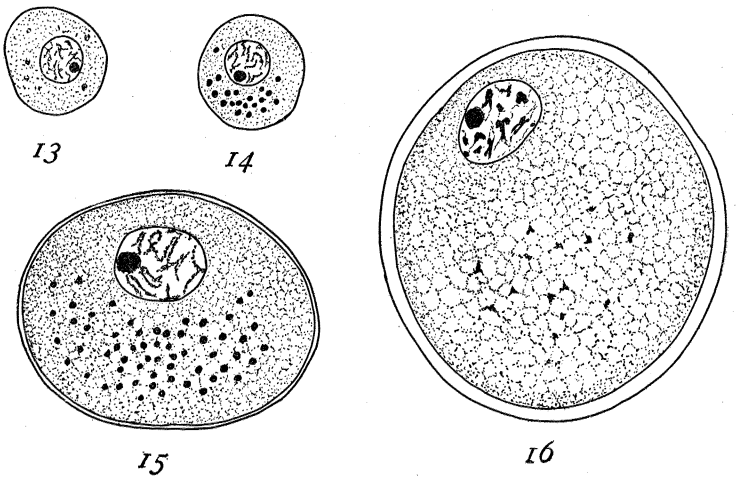


FIG. 13. A primordial ovocyte (×410).

FIG. 14. An ovocyte at the time when an epithelial follicle has just been established. Note the presence of yolk granules (×410).

FIG. 15. A half-grown ovocyte, showing a characteristic elongated shape and the presence of numerous yolk granules (×410).

FIG. 16. An ovocyte practically full grown, in the so-called "pseudoalveolar" stage. Note that the yolk granules have almost entirely disappeared (×410).

pinkish tint when subjected to Sudan III, a circumstance that would seem to indicate the presence of fatty materials in solution. Such a stage is evidently equivalent to that seen in *Dasyurus* and designated by Hill as the "pseudo-alveolar" stage (compare Hill, '10, Fig. 4). It will be noted that the nucleus is drawing closer to the periphery and has reached its maximum size, with a largest diameter of about .025 mm. and always somewhat flattened in form. The diameter of ovocytes of this type averages about .1 mm. The zona has attained its definitive thickness of .003 mm. and is a dense membrane showing no radiations like those which have given to the homologue of this structure in

other mammals the name "zona radiata." The cell is now over four times as great in diameter as the nucleus and there is little further alteration in their relative masses until after the rupture of the membrane of the germinal vesicle when the first maturation spindle is established.

During the stages of follicular development numbered 8, 9 and 10 there occurs a gradual reorganization of the cytoplasmic materials of the ovocyte. A zone of denser homogeneous protoplasm comes to occupy a peripheral position, forming an increasingly more and more sharply defined cortex, somewhat thickened at the point where the nucleus is flattened against the cell membrane. In the center of the ovocyte the protoplasm has assumed the character of a very coarse alveolar mass in the meshes of which are rather large irregular solid bodies which stain with Sudan III. The alveolar structure, which is seen in sections of material fixed in neutral formalin, assumes in paraffin sections the appearance of a fluid core in which are suspended scattered strands of deeply staining fibrous material and a central irregular mass of large solid pieces of irregular size and form. Such a condition finds its exact counterpart in *Dasyurus* (see Hill, '10, Fig. 1), and is characteristic of the same stages of follicular development. The condition at the time when the clearest definition is established between the two cytoplasmic zones is shown in Fig. 17. Here the "formative zone" (*f.z.*) is shown as a somewhat thicker cortical layer than is usually found, and the deutoplasmic mass (*d.z.*) is represented as coarsely alveolar, a structure which it appears to have when seen in formalin preparations. It will be noted that the nucleus occupies a position in the middle of the thickest part of the formative cortex, a position which probably represents the animal pole of the ovocyte. The diameter of the cell at this stage is on the average about .12 mm., while that of the nucleus has not changed since the stage represented in Fig. 15. The ovocyte is now full grown and ready for the changes incidental to the maturation processes.

During maturation a very radical change in the cytoplasmic structure of the ovocyte takes place. The comparatively homogeneous formative zone of the full-grown ovocyte has

moved to one pole and forms a cap of considerable density, thick in the center and thinned out at the periphery. The deutoplasmic mass (*d.z.*) occupies the opposite pole and is practically in contact peripherally with the ovocytic membrane. The structure of this deutoplasmic material is represented in

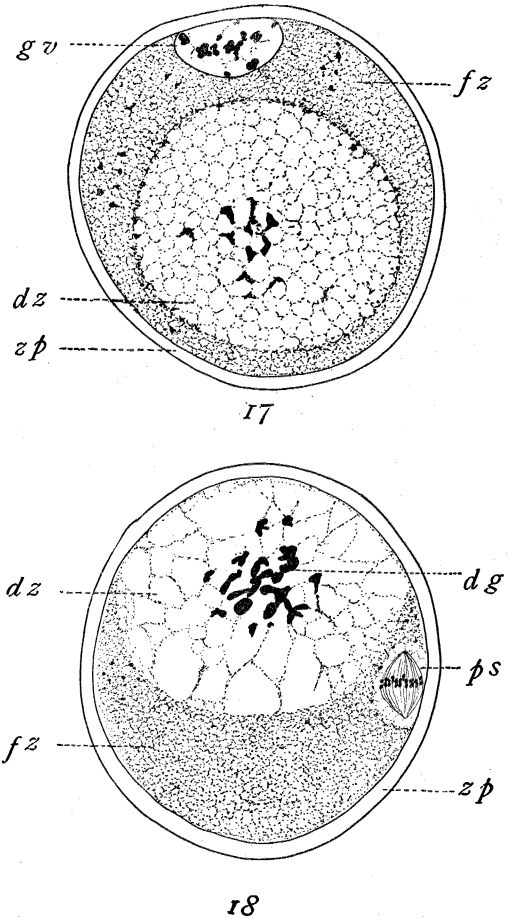


FIG. 17. A full-grown ovocyte, showing cytoplasmic organization, etc. Deutoplasmic zone (*dz*), formative zone (*fz*), germinal vesicle (*gv*), zona pelucida (*zp*) (×410).

FIG. 18. A maturing ovocyte, showing the new reversed polarity. The ovocyte is placed with the animal pole upwards. The deutoplasmic zone (*dz*) occupies the animal pole, the formative zone (*fz*) occupies the vegetative pole. The polar spindle (*ps*) lies in a tangential position at the equator of the ovocyte. Deutoplasmic granules (*dz*) lie in the center of the deutoplasmic mass. The zona pelucida *zp*) is a dense envelope, without radiations.

Fig. 18 as it appears in paraffin sections. The two zones are very sharply defined. The first maturation spindle, which one would expect to find in the thick middle part of the formative zone, occupies a position near the equator of the cell in the thinned-out peripheral portion of the formative zone that overlaps and partly surrounds the deutoplasmic mass. What is the significance of this peculiar position of the polar spindle? Exactly the same conditions are met with in the egg of *Dasyurus* and in that animal are interpreted by Hill as indications that the ovocyte has undergone a complete reversal of polarity. According to him the formative protoplasm occupies now the vegetative pole, while the deutoplasmic mass lies at the animal pole. This interpretation is borne out by the peculiar position of the spindle which occupies a position as near the animal pole as is possible without leaving the formative protoplasm or the peripheral position necessary for the extrusion of the polar body.

In view of these conditions we may well hesitate to apply any sort of phylogenetic interpretation to the "telolecithal" character of this ovum. Doubtless we have here a polarity of the germ cell which is merely incidental to changes connected with the extrusion of the deutoplasm, which I believe occurs in the armadillo in much the same fashion as that described by Hill for *Dasyurus*. The evidence for this conclusion forms the material for a subsequent paper. The armadillo ovum is to be considered as primitive not because it shows a "telolecithal" organization but because it is so nearly identical in many details with that of a number of marsupials.

With the rupture of the germinal vesicle and the establishment of the first cleavage spindle a very marked diminution in the size of the nuclear material is manifest. This is due to the loss of much fluid and perhaps some chromatin to the cytoplasm, and also to a marked condensation of the remainder, as will be shown more in detail when the nuclear phenomena come up for discussion.

VII. Nuclear Changes During the Growth Period of the Ovocytes.

The nuclear changes from the time when the ovocyte is in the primordial follicle stage till the establishment of the first cleavage spindle constitute the prophases of maturation. The earlier as well as the later changes should receive attention. Students of mammalian ovogenesis, however, have as a rule restricted their observations to the changes immediately connected with maturation divisions, ignoring the long period of nuclear and cell differentiation that leads up to and doubtless conditions these divisions. The following account makes no claim of comprehensiveness but will serve to suggest some of the more significant phases of nuclear behavior that are characteristic of this period.

The nucleus of the ovocyte in the primordial follicle (Fig. 19) shows the chromatin in the form of long, more or less coiled threads, which are sometimes so tangled as to form a pseudo-reticulum. In neutral stains such as iron hæmatoxylin and copper chrome hæmatoxylin the chromatin threads and the plasmosome take the same stain, but when double staining methods are employed the difference between the two materials is clearly brought out. When neutral safranin and acid violet are used the chromatin takes the violet color from the acid reagent and the plasmosome takes only the neutral color, appearing bright red. With toluidin blue and acid fuchsin the chromatin takes a red color from the acid dye and the plasmosome is stained bluish with the basic stain. With thionin and erythrosin the chromatin takes a red color from the acid erythrosin and the plasmosome is stained purple from the thyonin. Evidently then at this period the chromatin is basic and the plasmosome acid in character. The plasmosome is also shown to be a vesicular structure containing some vacuoles or granules. It is practically certain also that the chromatin threads, each of which must be identified as an elongated chromosome, are diploid in character, since synapsis has occurred during the organogenesis of the ovary. It is only in later stages that the diploid composition of these bodies manifests itself.

In the early simple epithelial stage of the follicle no marked

changes in the character of the nuclear elements has occurred (Fig. 20), except that the chromosome threads are a little shorter and thicker.

As the follicle develops through stages 2, 3, and 4 we find nuclear changes corresponding to those shown in Figs. 21, 22, and 23, which are evidently to be interpreted as stages in the separation of the closely fused diplotene threads into their component halves. Occasionally the whole complex, as in Fig. 21, is seen to be composed of elements distinctly double in character. More frequently, however, the condition is less obvious, as in Figs. 21 and 22, where the double elements have opened up into V's and rings, or occasionally have become precociously condensed into chromsomes of tetrad-like structure. There is no change as yet in the staining reactions of the nuclear components.

In connection with follicular stages 5 and 6 we customarily find in the nucleus of the ovocyte very marked shortening of the chromosomes accompanied by an increasing vagueness and irregularity of outline (Fig. 24). The diploid character of the elements is pronounced. The chromatin is undergoing a chemical change from a basic to an acid character, while the plasmosome still retains its acid affinities. This change is shown clearly when the neutral safranin and acid violet combination is used, for the chromatin no longer stains violet but assumes a faint pinkish hue from the safranin. Likewise, when toluidin blue and acid fuchsin are employed the chromatin stains bluish instead of bright red as formerly, indicating a change from basic to acid character. Slender threads of linin connect the various chromosome bodies. The chemical character of the plasmosome is not altered.

In follicular stages 7 and 8 the process of chromosome condensation has made considerable progress as is shown in Fig. 25. There are very great individual differences in the degree of condensation seen in the various units of the complex. Some of them have attained a form practically like that seen just before spindle formation, while others are still elongated and irregular. Linin threads, usually double in character, still form numerous connections between the chromatin elements. Both chromosomes and plasmosome at this stage take basic stains with about equal

avidity, the latter being easily recognizable from the former by its greater size and vesicular character.

In follicular stages 8 and 9 the nucleus assumes the character shown in Figs. 26*a* and *b*, two sections through the same nucleus. Here the entire chromatin complex has undergone further condensation until each element is in the form of a double chromosome more or less clearly defined. There is a strong tendency, often much more clearly manifest than the illustration would indicate, for the larger chromatin elements to aggregate into a dense central mass and for the smaller elements, which are frequently single spherical bodies, to lie in contact with the nuclear membrane. A few of these small peripheral elements are noted to be connected by linin threads with the large chromosomes forming the central group. It is not possible to enumerate the chromosomes at this time, but a number of fairly accurate approximations have been made which would indicate that there are about sixteen large chromosomes besides a varying number of small peripheral elements. These bodies may be supernumerary chromosomes which subsequently either attach themselves to the large elements or are thrown out into the cytoplasm on the rupture or dissolution of the nuclear membrane. There are seldom any evidences of small chromosomes in the spindles during the actual maturation process, but partially formed spindles, as that shown in Fig. 29, show certain small elements attached to spindle fibers which may be homologized with the small peripheral chromatin elements of the stage of nuclear development under consideration. The plasmosome is no longer to be identified with certainty. In Fig. 26*b* is seen a large body of diploid form which stains less deeply than do the other chromosomes. This may be a stage in the transformation of the plasmosome into a chromosome, but of this I cannot be certain. In none of the nuclei examined have I seen indications of the dissolution of the plasmosome and am inclined to the view that it is the equivalent of a heterochromosome. Another point which is obvious in the figures is the increasing irregularity of the nuclear membrane. It is evidently becoming very thin and is losing its turgor, as the wrinkles in its surface indicate. This could hardly be due to shrinkage in fixation, for the nuclei of earlier stages retain their

spherical contours. Moreover the wrinkles occur chiefly on the side of the nucleus toward the cell membrane to which is in close proximity at this time.

In follicular stage 10 the nuclei of ovocytes are in a condition represented by Figs. 27*a* and *b*. These two drawings indicate the entire chromatin content of a single nucleus. It will be noted that the chromosomes are clearly defined but still massed in groups. There is no distinguishable plasmosome. All of the chromatin takes with equal avidity basic dyes and must therefore be considered rich in nucleinic acid. There are only remnants of the linin network in the form of scattering slender threads running from the chromosomes to the periphery of the nucleus. A number of these threads appear to converge at one point and may indicate the first steps in spindle formation. Subsequent stages deal with the actual maturation divisions.

VIII. The First Polar Spindle.

The stages immediately preceding the establishment of the complete cleavage spindle are difficult to find in sections, owing probably to the great rapidity of the process. One of the few stages that have come to light is shown in Figs. 28*a* and *b*. In this case it is obvious that the spindle is forming, but chiefly at one end. The chromosomes are very clearly defined and have lost the tendency to be grouped into masses. There is a marked difference in the size of the individual elements, some being many times the volume of others. This is interesting in view of the fact that in the fully formed spindle there is no marked size difference among the chromosomes. The explanation of this condition probably lies in the inequality of state of condensation in the various elements. Numerous camera drawings of the chromosome complex of this period have been made, and on the basis of such drawings chromosome counts have been attempted. It is extremely difficult, however, to get even approximately correct estimates of the actual number of univalent and bivalent elements present. It seems quite obvious from examination of the figures that both single and double elements occur side by side, and it is not always possible to distinguish one type from the other. A study of Figs. 28*a* and *b* will reveal some elements

very clearly tetrads and others that are clearly bivalent though not as yet tetrapartite. The small peripheral chromosomes of earlier stages are still in evidence. Some of these show no tendency to take a position in the spindle and are evidently destined as a contribution of the nucleus to the cytoplasm on the dissolution of the nuclear membrane. That this extrusion of chromatin into the cytoplasm actually occurs is evidenced by the presence in the cytoplasm of mature ova of minute nucleus-like bodies of cytoplasmic chromatin.

The spindle is evidently completely established within the nuclear membrane as one must conclude from the occasional occurrence of such appearances as that shown in Fig. 29. In this case the membrane is exceedingly delicate but still unmistakable. When the membrane finally disappears there is evidently cast out into the cytoplasm a large amount of material, largely fluid, but probably partially solid in character. With the loss of this liquid the spindle shrinks in size and the chromosomes undergo marked condensation, as must be evident from comparison of these elements in Figs. 29 and 30 which are drawn to the same scale. Still further condensation of chromatin seems to occur during the metaphase as a comparison of Fig. 34 will show. The late prophases show the chromosomes as bivalent elements, occasionally having the appearance of typical tetrads (Figs. 31 and 32). The spindle is apparently a naked central spindle without mantle fibers or asters and is evidently a self-contained system insulated from the cytoplasm by a sheath of inert material, coarsely vacuolated, a material which may consist largely of the extruded nuclear sap which is in equilibrium with the surrounding cytoplasm, and through which no metabolic exchanges between nucleus and cytoplasm can occur.[1]

In equatorial plate views of the metaphase one can frequently count the chromosomes with every assurance of accuracy. Fig. 32 is a typical equatorial plate view of the first maturation spindle and one can judge from this example as to the feasibility of enumerating the chromosomes. By far the most frequent count obtained is that of 16 diploid elements, but some counts show as few as 14

[1] These conditions accord with those described by F. R. Lillie for certain phases of maturation in the egg of Nereis.

and others as high as 19. The larger number may be, and probably is, due to the precocious separation of a few of the double elements in the early anaphase. The lower count may be due to some elements being fused with or hidden under others. Personally I am convinced that the normal reduced number of chromosomes in the female of this species is 16, since this number has occurred as often as all others combined.

Fig. 34 shows a rather unusual spindle in which all of the chromosomes are in the form of well-defined tetrads, in some cases just separated into diads. A number of other spindles have been found where some of the elements were typical tetrads of this sort, but others were merely double in appearance. In the anaphases, a good view of which is given in Fig. 35, the chromosomes are clearly diads of dumbell shape. In late anaphases, as in Fig. 36, these diads assume the flattened shape characteristic of the tetrads of the prophase and form ring- or disc-shaped masses at either end of the barrel-shaped spindle. The band-shaped *Zwischenkörper* forms a characteristic feature of this phase of the division.

During the prophases and anaphases of polar body formation the spindle lies in a parallel or tangential position with reference to the cell membrane and it is only in the last steps of the anaphase that any sign of polar extrusion appears. The polar body begins to form after the manner shown in Fig. 36, by the appearance of a slight furrow, like the beginning of a cleavage furrow. As this furrow deepens the spindle assumes a position more nearly perpendicular to the surface of the ovocyte and the polar body is constricted off as in Fig. 37.

IX. The First Polar Body and the Second Polar Spindle.

Only eight ovocytes with one polar body have been found in the present study as compared with literally hundreds with first polar spindles. This fact would seem to suggest that the majority of the ovocytes come to an equilibrium in the metaphase of the first maturation division, and require some special stimulus to cause them to complete this division. It is very probable that the stimulus needed is that brought about by mating. If we may infer from analogy with other mammalian studies of ovulation

it is likely that follicular rupture takes place immediately after the ovocyte has extruded one polar body; hence all of the cases observed in which one polar body has been formed must be considered as ovocytes ready for ovulation. As a rule the first polar body as it appears in ovarian ovocytes is much compressed between the zona and the cell membrane, as in Figs. 38 and 41, and is some distance from the second maturation spindle. This position is, I believe, not to be interpreted as due to a migration of the ovocytic nucleus in the interim between the two maturation divisions, but rather as a shifting of the freed polar body. Observers of living mammalian ovocytes, notably Long and Mark ('11), indicate that there is a considerable space between the ovocyte membrane and the zona after the first polar body is extruded. This space would offer the necessary conditions for any shifting in the position of the polar body with reference to the site of the maturation spindle. In Fig. 40 is shown a case where the second polar spindle has established itself in close proximity to the first polar body, in which the nucleus has remained in a comparatively solid state. This is not a usual condition, however, for in practically all other cases the first polar body shows signs of a tendency to undergo mitotic division. I have never observed a well-defined spindle in a polar body, but radiating fibers are always present and the chromosomes to some extent divide and pass to two poles of the cell, as in Fig. 38. In only one case have I observed a complete division of the first polar body and that is in a decidedly atypical case shown in Fig. 42 and which is discussed in a subsequent connection. The division of the first polar body must then be considered as merely an abortive attempt at a division equivalent to the second maturation division. It seems likely that the chromatin subsequently goes back into a vesicular nucleus like that shown in Fig. 40, or like that in the fertilized egg shown in Fig. 44.

The second polar spindle can be identified with assurance only in ovocytes in which the first polar body can be observed, and as was indicated, these conditions are comparatively rare. Yet we have several exceptionally good examples of such spindles, notably those shown in Figs. 38, 39, 40 and 41. As a rule the second spindle is noticeably smaller than the first, but the dif-

ference is not nearly so obvious as is the case in the mouse egg. Apart from the lack of typical tetrads in the second spindle the chromosomes have the same general appearance as those of the first. As an illustration of this similarity of chromosomes compare Figs. 32 and 39, which are equatorial plate views of the chromosomes of first and second polar spindles respectively. The spindle shown in Fig. 41 differs from any other, either first or second, that has come under my observation in that it appears to have a set of mantle fibers in addition to the central spindle. This, however, is not to be considered typical for second maturation spindles in this species.

All of the conditions thus far described have been found in follicles of normal structure, in which there are no evidences of follicular atresia, and are, therefore, to be considered as strictly normal.

X. The Second Polar Body and the Female Pronucleus.

My observations of the second polar body are limited to three cases, the anomalous case described on the following page and illustrated in Fig. 42; the normal case shown in Fig. 43; and the fertilized egg, Fig. 44. The normal case from which the drawing (Fig. 43) was made occurred in a follicle like that shown in follicular stage 11. The section cuts tangentially across one part of the formative zone and just shaves a thin slice from the deutoplasmic mass, shown in the form of coarse vacuoles. The second polar body appears to be somewhat larger than the first, in this respect resembling the egg shown in Fig. 42. The chromosomes of both polar bodies are well scattered and there are evidences of an attempt at mitotic division. The female pronucleus is in a condensed condition, but the individual chromosomes are distinguishable. Surrounding the mass of chromatin is a capsule of homogeneous protoplasm, which probably insulates the nucleus from its own cytoplasm.

XI. An Anomalous Case of a Third Polar Spindle.

This case came to my attention very early in the investigation and was at first interpreted as a practically certain case of a parthenogenetic first cleavage. In view of recent discoveries

of many examples of parthenogenetic cleavage it now appears that this spindle in no way resembles a cleavage spindle, for the egg is not in a condition for cleavage, in that the deutoplasmic mass is still an integral part of it. The only alternative explanation that occurs to me is that we have here a rare case of a continuation one step further than is normal of the processes involved in maturation. The spindle in this egg is as perfect as any polar spindle observed in my material and contains without question 16 chromosomes. It is a naked central spindle without any traces of the aster radiations characteristic of true cleavage spindles. As though in physiological sympathy with the egg cell both polar bodies are seen to have proceeded somewhat further in their development than those in any other egg observed. The first polar body has completely divided into two ootids, and the second polar body, which is very large and well-formed, shows a polar view of a mitotic spindle, homologous with that seen in the egg itself, and has likewise 16 chromosomes. This curious egg was found in a follicle like that shown in Fig. 12, in which the process of atresia had made noticeable progress. The case is unique in the annals of biology and may possibly be explicable on some other basis than that which I have suggested. Personally I see no alternative explanation.

XII. Fertilization.

Although diligent search has been made through large numbers of ovaries with fallopian tubes attached, only one tube egg has been found. This one egg, however, is so evidently a normal example of the conditions typical for the species that it warrants a detailed description. The egg is found in a part of the fallopian tube just where it straightens out in its course toward the uterus. It lies free in the tube surrounded by a coagulum of material evidently composed of the disintegrated fragments of granulosa cells. It runs through twelve serial sections of 10 microns thickness and is therefore about .12 mm. in diameter, or a little smaller than the average full grown ovarian ovocyte. The zona is well defined and unbroken. The two polar bodies, each with its nucleus in a resting phase, are situated in contact with the formative protoplasm at some distance from the pronuclei.

The male and female pronuclei are in contact and are contained within the main body of the formative protoplasm and are closely similar in size and in the condition of their chromatin, which is peculiar in that each chromosome appears to be a small vesicle, connected with others by means of linin fibers. A large plasmosome is present in each pronucleus. The cytoplasm does not show so clear a demarkation between formative and deutoplasmic zones as is usually seen in the maturation stages, but this is probably due to the fact that the plane of section is equatorial and therefore unfavorable for showing polar differentiation of any sort.

This account of a single case of fertilization might be considered a somewhat meager basis for an account of so important a process, but it is the best that can be offered at present. It is my conviction that the discovery of even one such stage is a fortunate circumstance, in view of the fact that we are dealing with wild animals captured at night and not operated on until the following morning at the earliest. Only rarely is it possible to obtain the females so soon after capture as this. The uterus associated with the ovary in which this fertilized egg was found was somewhat swollen and congested and was supposed to contain an early blastodermic vesicle, but on examination was found to be non-pregnant. No other ova were found in the fallopian tube, either proximal or distal to the cite of the one under discussion. There is a single medium-sized corpus luteum. These facts demonstrate that only one ovum is given off and fertilized at one time, and add confirmation to the contention that during the early stages of embryonic development in the armadillo the conditions are those of a single fertilized egg and a single blastodermic vesicle and that the separation into four embryonic rudiments is a process of asexual multiplication, whose *visible* manifestation comes comparatively late in the development of the blastodermic vesicle.

Summary and Conclusions.

1. A study of the ovogenesis of the armadillo reveals nothing unique except that the cytoplasmic polarity of the mature ovocyte and its genesis is practically identical with that of the

marsupial *Dasyurus*. In the sense that the ovum is like that of a member of a lower sub-class of mammals it may be considered as probably the most primitive Eutherian ovum on record. The deutoplasmic material is at first centrally situated within a capsule of formative protoplasm, but, coincident with the onset of maturation, a shifting of materials takes place, so that the deutoplasmic material is aggregated at the animal pole of the ovocyte while the formative protoplasm forms a cap-shaped mass at the vegetative pole. The first polar spindle occupies a position as near the animal pole as it can without leaving the surface of the cell or the formative material. The earlier condition may be called "centrolecithal" and the later, "telolecithal," though these terms probably imply homologies that do not exist.

2. Previous studies of mammalian ovogenesis are confined to three orders of mammals, Rodentia, Cheiroptera and Carnivora, all of which are rather highly specialized orders, according to modern systems of classification. The present study of conditions in the armadillo is the first contribution to our knowledge of the germ cells of the Edentata, and thus we may add not only a new order to the short list of those studied, but probably the order showing the most primitive conditions.

3. Since it had not been found feasible to breed the armadillos in captivity our knowledge of the maturation processes depends entirely upon studies of the ovarian ova, normal and atretic. Nine tenths of the process as here described takes place in normal follicles and hence must be considered as strictly normal. A large number of ovaries have been sectioned in order to secure the stages described in the present history. Abundance of material is necessary because some of the stages are of exceedingly rare occurrence.

4. A study of the developmental history of the follicle shows that there is no basis for the idea expressed by Rosner that the four embryos are derived from the fusion of four adjacent follicles and the coöperation of their four ova to form a compound blastodermic vesicle.

5. The full grown ovum is about .12 mm. in diameter. It is smaller than that of the cat and larger than that of man.

6. The first polar spindle and first polar body are in no way radically different from those described for other mammals. The

chromosomes appear to afford exceptional opportunities for enumeration. The haploid number appears to be 16 and the diploid, 32. There are numerous apparent exceptions, but these are the most commonly appearing numbers.

7. The second polar spindle and second polar bodies are of rare occurrence, but those studied are in no way different from those of other mammals.

8. An exceptional case of what appears to be a third maturation division is figured and discussed.

9. A single tube egg in a good state of preservation shows a late stage in the fertilization process. Both polar bodies are present, and the male and female pronuclei are large vesicles practically ready for fusion. This one case is adjudged to be typical and, on the basis of its discovery, we are in a position to add another item to the evidences already published that the quadruplets of the nine-banded armadillo are derived from a single fertilized egg. The case also serves to eliminate from further consideration all suggested explanations of the underlying basis of polyembryony that involve the idea of polyspermy.

HULL ZOÖLOGICAL LABORATORY,
UNIVERSITY OF CHICAGO,
May 1, 1912.

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Explanation of Plates.

Plate I.

Figs. 19 to 25 (inclusive) show seven successive stages in the development of the nucleus of ovocytes during the growth period (× 1,600).

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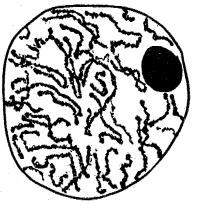

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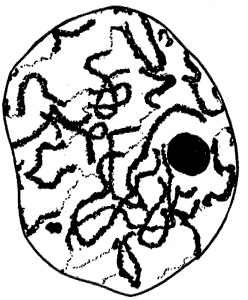

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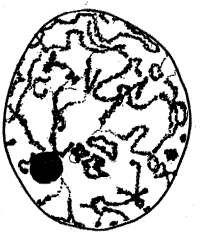

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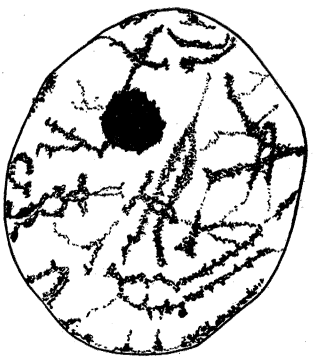

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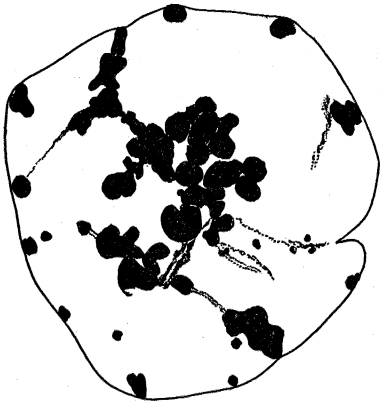
H. H. NEWMAN.

PLATE II.

FIGS. 26 (*a* and *b*) represent two sections through the germinal vesicle of an ovocyte in a follicle like Fig. 9. (×1,600).

FIGS. 27 (*a* and *b*) show two sections through the germinal vesicle of an ovocyte in a follicle like Fig. 9 (×1,600). Note the first indications of spindle fibers.

FIGS. 28 (*a* and *b*) show two sections through a germinal vesicle of an ovocyte like that represented in Fig. 11. Note that considerable progress has been made in the establishment of the first polar spindle and that the bivalent chromosomes are of very unequal size and form, some having a distinct tetrad form (×1,600).



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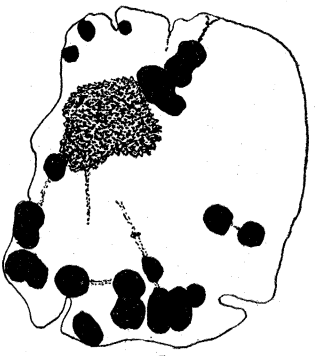

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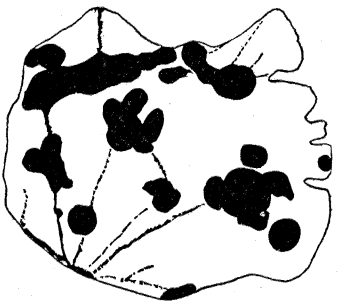

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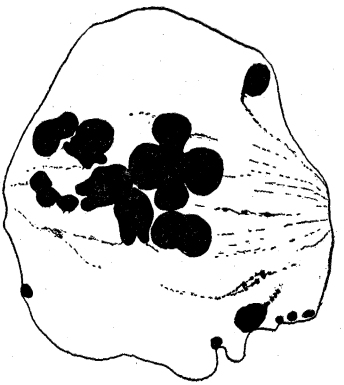
27 b

28 a



28 b

PLATE III.

FIG. 29. A newly formed first polar spindle still within the membrane of the germinal vesicle (×1,600).

FIGS. 30 and 31. Two first polar spindles in late prophases (×1,600).

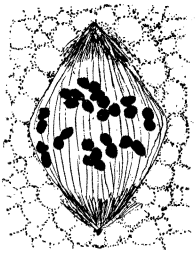
FIG. 32. An equatorial plate view of the chromatin complex of a metaphase of the first polar spindle (×1,600).

FIG. 33. A portion of an ovocyte showing a diagonal section through the equatorial plate of a metaphase stage of a first maturation spindle. Note the capsule of hyaline protoplasm surrounding the spindle, insulating it from the cytoplasm (×800).

FIG. 34. An unusually fine first polar spindle in the metaphase or very early anaphase. The chromosomes are all typical tetrads (×1,600).

FIG. 35. A middle anaphase of a first polar spindle, showing the diads as dumbbell-shaped bodies. Faint indications of centrosomes are visible. Compare the shape of the diads with that of those in the next figure (×1,600).

FIG. 36. A late anaphase of a first polar spindle. Note the changed shape of the diads, the bands-like *Zwischenkörper*, the insulating protoplasmic capsule divided into two, and the cleavage furrow destined to cut off the polar body. The position of the spindle with reference to the periphery of the ovocyte is typical (×1,600).



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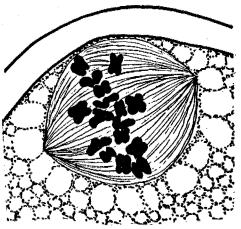

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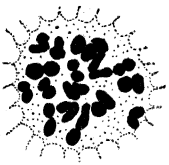

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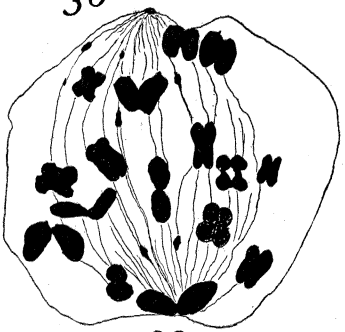

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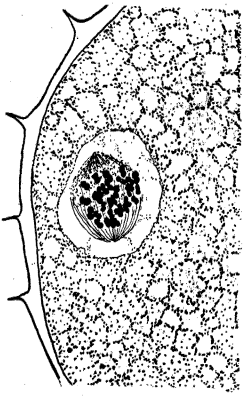

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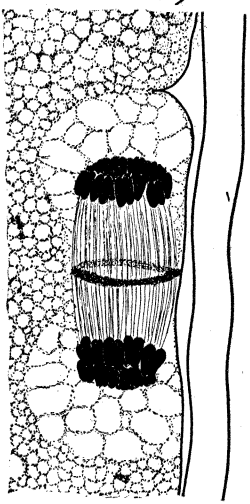

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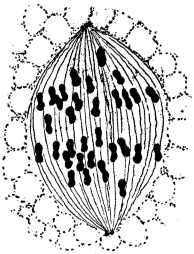

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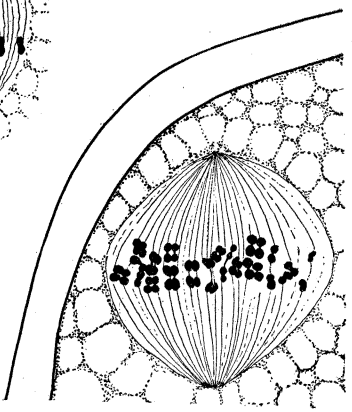

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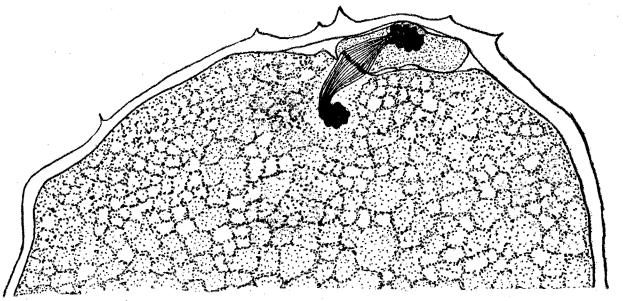
PLATE IV.

FIG. 37. Portion of an ovocyte showing the formation of the first polar body (×800).

FIG. 38. Portion of an ovocyte showing the first polar body undergoing an abortive attempt at division, and an equatorial plate view of the second polar spindle (×800).

FIG. 39. A high power drawing of the chromosome complex of the second maturation spindle shown in Fig. 38 (×1,600).

FIG. 40. Portion of an ovocyte with first polar body in a resting state and a good side view of the second polar spindle (×800).



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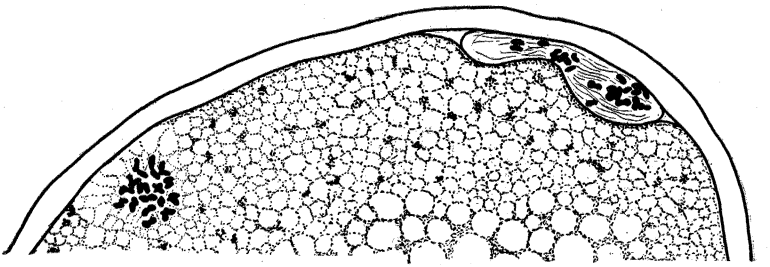

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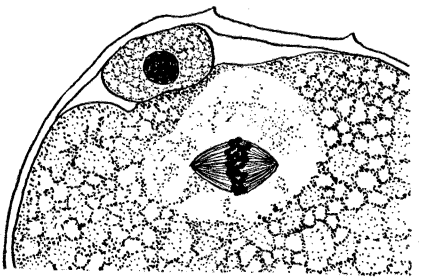

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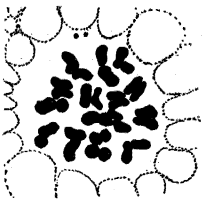

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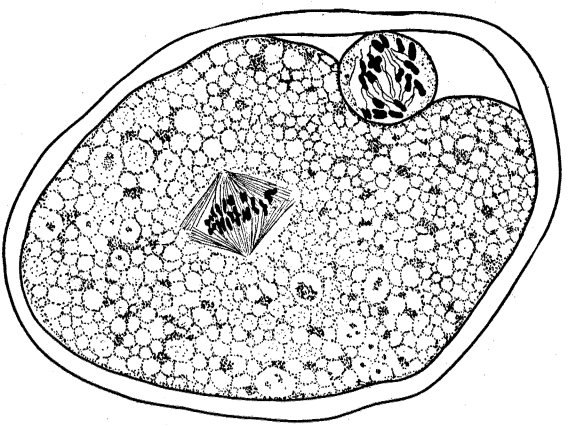
PLATE V.

FIG. 41. Section through an ovocyte with a dividing first polar body and a second polar spindle of unusual character, with mantle fibers. The spindle is in an early anaphase (×800).

FIG. 42. A section through one end of an ovocyte showing three polar bodies and a third maturation spindle. The first polar body has divided, the second polar body is dividing. The egg is somewhat flattened by shrinkage but the zona pelucida is intact (×800).



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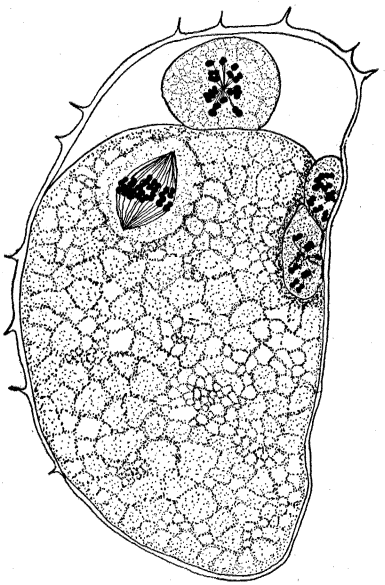

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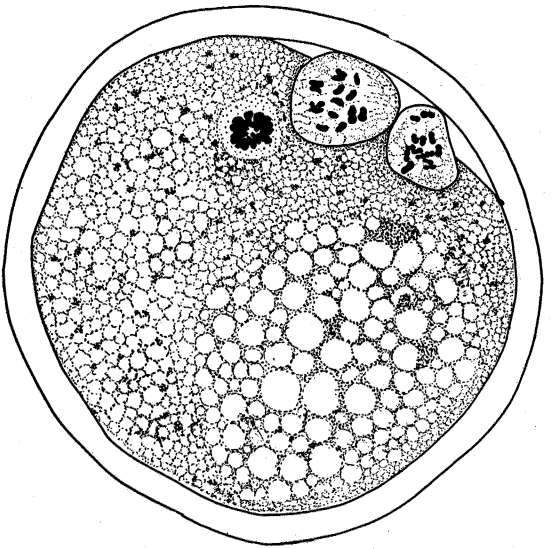
PLATE VI.

FIG. 43. A section through a mature egg with two polar bodies and a resting female pronucleus (×800).

FIG. 44. View of a fertilized egg found in the fallopian tube. Note the two polar bodies with their resting nuclei and the male and female pronuclei in contact, with their chromosomes in a vesicular or reticular condition. The drawing is a reconstruction of three sections through the formative protoplasmic cap (× 800).



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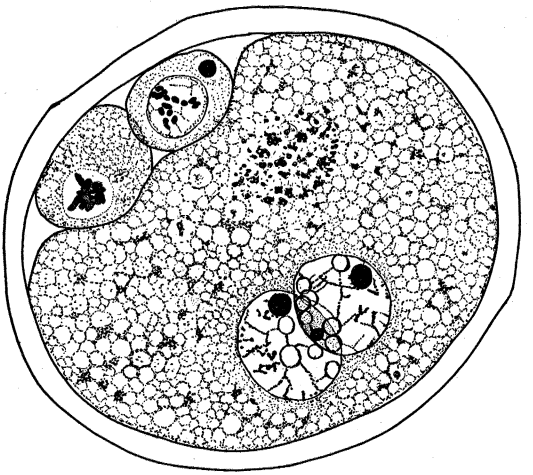

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